PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 166, 153–156 (1981)

Copper Metabolism of Cultured Fibroblasts from the Brindled Mouse (Gene Symbol *Mo*^{br}) (41038)[1]

ATEF K. SAYED, JOHN A. EDWARDS, AND ROBIN M. BANNERMAN

Medical Genetics Unit, Department of Medicine, State University of New York and the Buffalo General Hospital, 100 High Street, Buffalo, New York 14203

Abstract. The metabolism of copper by cultured fibroblasts from brindled male (*Mo*^{br}/Y) and normal male (+/Y) mice was studied using radioactive copper (^{64}Cu). Brindled fibroblasts accumulated more ^{64}Cu than normal fibroblasts. The accumulation of ^{64}Cu by both brindled and normal fibroblasts was enhanced if the cells were preincubated in a copper-supplemented medium or if fetal calf serum was omitted from the medium. Brindled fibroblasts also retained more ^{64}Cu on continued incubation. The greater accumulation might, therefore, represent an effect of reduced copper efflux. The demonstrated expression of the inherited defect in copper metabolism of brindled mice in cultured fibroblasts emphasizes the value of this model for further study of copper metabolism and its genetic control.

The alleles at the mottled locus (*Mo*) on the X-chromosome of mice are manifested by lethal and sublethal effects in hemizygous males and mosaic coat color in heterozygous females. In order of increasing severity of effect, these alleles are blotchy (*Mo*^{blo}) (1), viable brindled (*Mo*^{vbr}) (2), brindled (*Mo*^{br}) (3), dappled (*Mo*^{dp}) (4), and tortoise (*To*) (5). Male hemizygotes which survive beyond birth show severe dilution of coat color, and altered hair structure with curly whiskers. Brindled (*Mo*^{br}) hemizygous males show a neurological disturbance, and marked reduction in serum ceruloplasmin and tissue copper, except for the small intestine which has a high copper content (6).

In Menkes' X-linked kinky hair disease (7, 8) there are similar but not identical clinical and biochemical abnormalities (6). Because of this resemblance, and to study the gene effect *in vitro*, copper metabolism of cultured fibroblasts from brindled male mice was investigated (9, 10).

Materials and methods. The *Mo*^{br} allele in our colony originated in mice of mixed stock from D. S. Falconer's colony in Edinburgh given to one of us (R.M.B.) in 1963. It is now maintained in an inbred line approaching the C57BL/6J by repeated backcrosses.

Primary skin explants were obtained from 4-day-old hemizygous brindled and normal littermate male mice. The explants were placed in minimum essential medium (MEM; 109 G Gibco), supplemented with 20% fetal calf serum (FCS) and 100,000 units/ml kanamycin. The copper content of the FCS was 25 μg/100 ml by direct determination (11) and no ceruloplasmin could be detected by the *O*-dianisidine dihydrochloride method (12). All cultures were grown in 30-cm^3 flasks incubated at 37° in a humidified atmosphere and 5% CO_2, unless indicated otherwise. When the primary cultures reached confluency 10–14 days later, the first subculture was carried out. Further subcultures were carried out twice weekly. After 3 weeks (i.e., six or seven passages), and on reaching confluency, the copper experiments were initiated.

Cellular copper accumulation was investigated by plating the established cultured fibroblasts in microplates (Linbro FB-16-24-TC) in 2 ml of the above medium. Each culture was seeded in duplicate. Then, 36–42 hr later, and on reaching confluency, the medium was replaced by medium containing 0.01 mmole ^{64}Cu as $^{64}Cu(NO_3)_2$ (sp act 4.02 mCi/mg, New England Nuclear,

[1] This work was supported by Grant 1 R01 AM19424 from the National Institutes of Arthritis, Metabolic and Digestive Diseases, and Human Genetics Program Project 417 of HEW Department of Maternal and Child Health, and presented in part at the annual meeting of the Society for Experimental Biology and Medicine, Atlantic City, New Jersey, April 1978.

0037-9727/81/010153-04$01.00/0

Boston). All cultures were incubated at 37° in a closed system using adhesive sealers (Linbro-64-PSM). At intervals of 1, 4, 8, 12, and 21 hr harvesting was initiated by discarding the radioactive medium, and washing the cells twice with 0.2 mmole/liter EDTA in isotonic saline. The cells were lysed in 0.1 *N* NaOH. The radioactivity in the lysate was measured in a gamma well counter. The protein content of the lysate was measured (13, 14) and the results were expressed in counts per minute per milligram of protein.

In order to exclude any possible effect of cellular copper deficiency, established cultured fibroblasts from brindled and control mice were seeded in duplicate in microplates in 2 ml of medium with added copper (+Cu) as $CuSO_4$ to a concentration of 0.05 mmole/liter Cu^{2+} (i.e., three times the physiological serum copper concentration). Duplicate cultures without added copper were set up in parallel. Then 36–42 hr later the medium was replaced by fresh medium containing ^{64}Cu in MEM with or without 20% FCS and cellular ^{64}Cu accumulation was determined as described above.

For the study of copper release, the established cultured fibroblasts were transferred to 75-cm^3 flasks in duplicate. After 36–42 hr and on reaching confluency, the culture was continued for 12 hr in FCS-supplemented MEM containing ^{64}Cu. The radioactive medium was then discarded, and the cultured cells were washed twice with EDTA solution and incubated with fresh medium. The cells were harvested at 0, 4, 8, and 16 hr, and the remaining intracellular ^{64}Cu was determined as described above. The results were expressed as a percentage of the radioactivity present at zero time.

Results. *Copper accumulation.* The cultured fibroblasts from brindled mice accumulated ^{64}Cu faster than those of control male mice (Fig. 1). From the first hour, the brindled cells incorporated more ^{64}Cu than the normal cells and within 21 hr, the accumulation by brindled cells appeared to be reaching a plateau. The mean accumulation values for 21 hr were 6800 cpm/mg protein (range 6000–7600) for cells from four brindled male mice, and 3000 cpm/mg protein

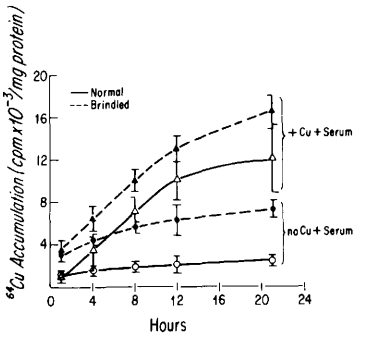

FIG. 1. The effect of preincubation with copper on the ^{64}Cu accumulation (cpm × 10^{-3}/mg protein) by normal and brindled cultured fibroblasts in the presence of fetal calf serum.

(range 2200–3800) for cells from eight controls.

Both normal and brindled fibroblasts accumulated more ^{64}Cu when they were preincubated in a copper-rich medium (Fig. 1). The mean ^{64}Cu accumulation at 21 hr under these circumstances was 17,200 cpm/mg protein (range 15,400–18,400) by brindled fibroblasts, and 11,200 cpm/mg protein (range 6400–16,000) by normal fibroblasts. The effect of added copper on the ^{64}Cu accumulation by both brindled and normal was enhanced when fetal calf serum was omitted from the radioactive medium during the incorporation experiment (Fig. 2). At 21 hr the brindled cells accumulated twice as much as the normal cells (38,000 vs 15,600). When both cell types were preincubated in the absence of added copper and the incorporation experiments were carried out in the absence of fetal calf serum, the brindled cells again accumulated more ^{64}Cu than the normal cell (Fig. 2). The mean 21-hr values were 12,800 cpm/mg (range 10,000–15,600) for brindled cells, and 4400 cpm/mg (range 3400–6400) for normal cells.

Copper release. At 4 and 8 hr after replacement of ^{64}Cu-containing medium by nonradioactive medium the brindled cells retained 55 and 49% of the radioactivity, whereas the normal cells retained 27 and 19%, respectively (Fig. 3). The greatest loss of ^{64}Cu by both brindled and normal cells occurred within the first 4 hr.

Discussion. The present studies demon-

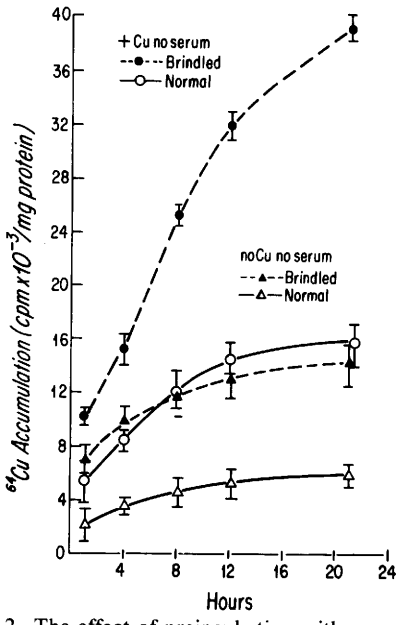

FIG. 2. The effect of preincubation with copper on the ^{64}Cu accumulation (cpm $\times$ 10^{-3}/mg protein) by normal and brindled cultured fibroblasts in the absence of fetal calf serum.

strate that cultured fibroblasts from brindled hemizygous male mice accumulate excessive amounts of radiocopper. The mechanism for this excessive accumulation is not clear, but it must be due to increased influx, decreased efflux, or a combination of both. In the absence of observations on initial rates of copper uptake by brindled cells, it is not possible to determine with certainty whether copper influx is increased. The present findings of diminished release of radioactive copper from cultured fibroblasts from brindled mice are suggestive of diminished copper efflux. This interpretation remains uncertain, however, because the absolute copper content of the cultured cells was not measured. Hence the possibility of increased dilution of the influxing radioactive copper by an enlarged intracellular pool of nonradioactive copper cannot be excluded.

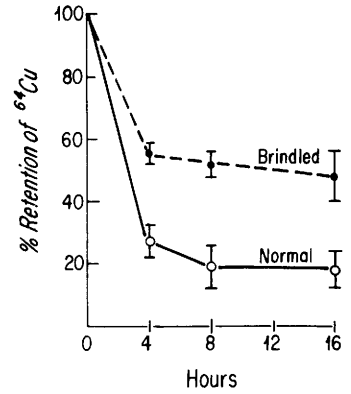

FIG. 3. Release of ^{64}Cu from normal and brindled cultured fibroblasts previously incubated in ^{64}Cu-containing medium, expressed as a percentage of the amount of ^{64}Cu accumulated initially.

When brindled and normal fibroblasts were grown in a copper-supplemented medium, their subsequent accumulation of radiocopper was enhanced, suggesting that the high accumulation of radioactive copper by brindled fibroblasts is not the result of cellular copper deficiency. It is not known why preexposure of cultured fibroblasts to copper enhances their subsequent accumulation of radiocopper; it is possible that copper stimulates the production of an intracellular copper binding or storage protein.

When fetal calf serum was omitted from the incubation medium, the accumulation of radiocopper by both brindled and normal fibroblasts was enhanced, suggesting that some constituent of fetal calf serum might have been binding radiocopper and, hence, diminishing the amount available to the fibroblasts. There is no demonstrable ceruloplasmin present in the fetal calf serum, so some other constituent, such as albumin, might be acting as a binding agent.

The present studies demonstrate that the abnormality of copper handling in brindled mice is expressed in cultured fibroblasts by excessive accumulation of radiocopper. Similar abnormalities have been observed in cultured cells from human subjects with Menkes' disease (15–18). Danks has alluded to but not published similar observations in cultured cells from brindled mice (19). He and his co-workers have also noted the enhancing effect of increasing copper concentration of the culture medium on copper accumulation, which we also observed. He has suggested that the basic defect may reside in an intracellular

copper-binding molecule with abnormal, increased affinity for copper (18, 19), possibly a metallothionein as suggested by *in vitro* studies of Menkes' disease cells (20).

Continued investigation of these genetic defects which are expressed in cultured cells should provide further information about the genetic control of copper metabolism, and insight into the basic mechanism of Menkes' disease.

1. Russell, L. B., and Saylors, C. L., Genetics **47**, 7 (1962).
2. Cattanach, B. M., Pollard, C. E., and Perez, J. N., Genet. Res. **14**, 223 (1969).
3. Fraser, A. S., Sobey, S., and Spicer, C. C., J. Genet. **51**, 217 (1953).
4. Phillips, R. J. S., Genet. Res. **2**, 290 (1961).
5. Falconer, D. S., Indukt. Abstamm. Vererb Lehre **85**, 210 (1953).
6. Hunt, D. M., Nature (London) **249**, 852 (1974).
7. Menkes, J. H., Alter, M., Steigleder, G. K., Weakley, D. R., and Sung, J. H., Pediatrics **29**, 764 (1962).
8. Danks, D. M., Campbell, P. E., Walker-Smith, J., Stevens, B. J., Gillespie, J. M., Blomfield, J., and Turner, B., Lancet **1**, 1100 (1972).
9. Sayed, A. K., Edwards, J. A., and Bannerman, R. M., Fed. Proc. **37**, 746 (1978) (Abstract).
10. Sayed, A. K., Edwards, J. A., and Bannerman, R. M., Clin. Res. **26**, 707A (1978) (Abstract).
11. Carter, P., Clin. Chim. Acta **39**, 497 (1972).
12. Schosinsky, K. H., Lehmann, H. P., and Beeler, M. F., Clin. Chem. **20**, 1556 (1974).
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Bid. Chem. **193**, 265 (1951).
14. Miller, G. L., Anal. Chem. **31**, 964 (1959).
15. Horn, N., Lancet **1**, 1156 (1976).
16. Goka, T. J., Howell, U., "Amer. Soc. Hum. Genet., San Diego, Calif.," p. 48A (1977) (Abstract).
17. Beratis, N. G., Price, P., LaBadie, G., and Hirschhorn, K., Pediat. Res. **12**, 699 (1978).
18. Camakaris, J., Danks, D. M., Ackland, L., Cartwright, E., Borger, P., and Cotton, R. G. H., Biochem. Genet. **18**, 117 (1980).
19. Danks, D. M., Inorg. Perspect. Biol. Med. **1**, 73 (1977).
20. LaBadie, G. U., Beratis, N. G., and Hirschhorn, K., "Amer. Soc. Hum. Genet., Minneapolis, Minnesota, Oct. 1979," p. 53A (Abstract).

Received January 16, 1980. P.S.E.B.M. 1981, Vol. 166.